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**Intracellular uptake of EGCG-loaded deformable controlled release liposomes for skin cancer**

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## ABSTRACT

Caucasian population groups have a higher propensity to develop skin cancer, and associated clinical interventions often present substantial financial burden on healthcare services. Conventional treatments are often not suitable for all patient groups as a result of poor efficacy and toxicity profiles. The primary objective of this study was to develop a deformable liposomal formulation, the properties of which being dictated by the surfactant Tween 20, for the dermal cellular delivery of epigallocatechin gallatein (EGCG), a compound possessing antineoplastic properties. The results demonstrated a significant ( $p \leq 0.05$ ) decrease in liposome deformability index ( $74 \pm 8$  to  $37 \pm 7$ ) as Tween 20 loading increased from 0 to 10 % w/w, indicating an increase in elasticity. EGCG release over 24-hours demonstrated Tween 20 incorporation directly increased release from  $13.7 \% \pm 1.1 \%$  to  $94.4 \% \pm 4.9 \%$  (for 0 and 10 % w/w Tween 20 respectively). Finally, we demonstrated DilC-loaded deformable liposomes were localised intracellularly within human dermal fibroblast and keratinocyte cells within 2-hours. Thus it was evident deformable liposomes may aid drug penetration into dermal cells and would be useful in developing a controlled-release formulation.

**KEYWORDS:** Skin cancer; deformable liposomes; dermal release; controlled release; elastic liposomes

## 1. Introduction

Skin cancer is an increasing public health problem particularly in developed countries [1]. Currently, 2-3 million non-melanoma skin cancers and 132,000 melanoma skin cancers occur globally each year [2]. The large number of cases diagnosed present as a substantial burden to healthcare services [3, 4, 2]. Despite the fact that the majority of skin cancers are treatable, malignant forms of the cancer results in over 9,000 deaths annually worldwide [5]. Current treatment approaches are limited to local surgery to remove the tumour in addition to topical treatments with cream formulations. However, surgical removal may not be suitable for all patients whilst topical therapies are often linked with poor patient compliance stemming from high dose frequency requirements and unpleasant side effects [6]. In addition, topical treatments may cause skin irritation, weeping, cracking and blistering causing discomfort and pain [7-9].

Strategies for cancer management are focused on chemoprevention and chemoprotection. Existing anticancer agents often demonstrate poor safety profiles in addition to unpleasant side effect profiles, and there is an urgent need for novel agents which are both efficacious and possess a limited toxicity profile to non-malignant dermal tissue [10-12]. One group of compounds that have gained interest recently as novel candidates for this purpose are flavonoids, naturally occurring chemicals abundantly expressed in food and drink, and in particular the green tea catechin, epigallocatechin gallatein (EGCG) which is increasingly being exploited for its chemoprevention properties [13-15]. EGCG has been found to affect specific biological processes that could be exploited as targets for the prevention and treatment of cancer [16], and has been demonstrated to possess properties associated with the induction of apoptosis [17], promotion of cell growth arrest by altering the expression of cell cycle regulatory proteins [17], activation of killer caspases and the

suppression of oncogenic transcription factors [18, 19, 15] and pluripotency maintaining factors [20]. However, the application of naturally occurring compounds as chemopreventative and chemoprotective strategies for skin cancer management has so far been received with limited success and this may be largely due to inefficient delivery systems and limited oral bioavailability of promising agents [21-23]. Consequently, to achieve maximum clinical efficacy, novel approaches are required to enhance compound bioavailability, of which dermal delivery is particularly promising.

The principle function of mammalian skin is to offer protection from environmental chemicals and xenobiotics [24]. The penetration of drugs across the skin is significantly inhibited by the skin's inherent barrier properties [25] thus there is a need to develop carrier systems to enhance penetrability. To fulfil this goal, when applied topically nanoparticle mediated delivery systems (e.g. microemulsions, liposomes, ethosomes, deformable liposomes and solid lipid nanoparticles), would benefit the direct dermal delivery of compounds across the stratum corneum [26-28]. Additionally, such nano-scale structures are capable of improving drug loading, enhancing systemic bioavailability, imparting a sustained release profile and allowing targeted drug delivery [29, 30]. Furthermore, the topical application of such carriers reduces the incidence of undesirable side effects arising from systemic administration and enhances systemic absorption of drugs after topical application with permeation enhancers which irreversibly disrupt the stratum corneum [29, 30]. Controversy however surrounds the use of conventional liposomes due to their large size preventing skin penetration [31, 28, 32, 33], Cevc et al., [34] demonstrated that modification of the chemical composition of the lipid bilayer so as to decrease its Young's modulus resulted in the formation of deformable liposomes. These are able to gain access to the viable epidermis by overcoming the physical constraints imposed by the stratum corneum by diminishing the membrane elastic energy required for the liposome to deform and fit through

an aperture size smaller than their original diameter following which reforming to their original shape [35, 36, 31]. By being able to change shape and volume at minimal energetic cost, these structures may penetrate across hydrophilic pathways of intact skin [37, 36]. Deformable liposomes often include additional components designed to make the membrane more liable to deformation and these are termed edge-activators, typically including surfactants such as Tweens, bile salts and Myrj [38]. The inclusion of this extra component destabilises the vesicle bilayers by reducing the amount of energy required to expand the interface allowing the liposome to become more elastic thus increasing the flux across the skin [38-40].

The primary aim of this study was to develop and characterise a deformable controlled release liposome formulation able to pass through the stratum corneum for targeting toward intracellular uptake into dermal cells. The objectives of the study were therefore to: (i) assess the impact of the edge-activator Tween 20 on liposomal formulation size; (ii) characterise resultant liposomes vesicle size, surface charge and encapsulation efficiency; (iii) quantify deformability of resultant liposomes using a deformability index (DI); (iv) assess release of EGCG; (v) assess the deformable liposome stability ; (vi) assess cellular toxicity of EGCG and (vii) assess intracellular uptake in human dermal fibroblast and keratinocyte cells.

## **2. Materials and methods**

### ***2.1 Materials***

Soy Phosphatidylcholine (PC) was obtained from Avanti Polar Lipids. Cholesterol (grade,  $\geq 99\%$ ), Tween 20 and EGCG (grade  $\geq 95\%$ , E4143) were obtained from Sigma-

Aldrich. All other reagents including methanol and chloroform were obtained from Fisher Scientific. Ultrapure water was obtained from a Milli-Q purification system (Millipore, Billerica, MA, US). Polycarbonate filter, pore size 50 nm, was obtained from Sigma-Aldrich (WHA800309). Human dermal fibroblasts (HDFa) isolated from adult skin and all cell culture reagents (Medium 106 and low serum growth supplement (LSGS) kit containing supplemented medium containing foetal bovine serum, 2 % v/v, hydrocortisone 1 µg/mL, human epidermal growth factor, 10 ng/mL, basic fibroblast growth factor, 3 ng/mL, heparin, 10 µg/mL; DMEM media supplemented with 1 % L-glutamine, 10 % FBS, 1 % Penicillin Streptomycin and 0.25% amphotericin) were obtained from Life technologies (Carlsbad, California, US). Immortalized human keratinocytes (HaCat) cells were a kind gift from Dr Andrew Sanders (Cardiff China Medical Research Collaborative, Cardiff University, Henry Wellcome Building, Heath Park, Cardiff, CF14 4XN). 4,6-diamidino-2-phenylindole (DAPI) and 1,1'-Diocetadecyl-3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiIc) was obtained from ThermoFisher Scientific (D1306 and D282 respectively).

## **2.2 Methods**

### *2.2.1 Preparation of deformable liposomes with or without an edge activator*

Liposomes were prepared by adapting the film hydration method established by Bangham *et al.*, (1965) [41]. Soy PC and cholesterol (16:8 µM) were dispersed in an organic solvent mixture consisting of chloroform and methanol in a 9:1 (v/v) ratio in a round bottomed flask [40, 38, 26, 42, 41]. Subsequently, the organic solvent was removed by rotary evaporation for 5 minutes at 35 °C, followed by purging with nitrogen gas. The resultant dry film residue was hydrated by the addition of 4 mL water containing edge activator (up to 10% w/w of the formulation) and 1 mg of EGCG at a temperature above the transition temperature of the phospholipid (between -7 to -15°C) [43] and vortexed for 5 minutes to form

multilamellar vesicles (MLV). The resulting particles were extruded 21 times through 100-nm diameter polycarbonate membranes, using an Avanti Mini Extruder to produce unilamellar vesicles. The formed liposomes were equilibrated for 30 min above their transition temperatures ( $-15^{\circ}\text{C}$ ) before being subjected to further characterisation [44, 45, 43].

### *2.2.2 Deformable liposome characterisation*

The mean particle size and the polydispersity index (measurement of the level of homogeneity of particle sizes) of liposomes were measured by dynamic light scattering (DLS) using a Zetaplus (Brookhaven Instruments) following dilution with distilled water (1:4 ratio) to ensure intensity adjustment. A polydispersity value of  $< 0.2$  indicates a homogenous vesicle population, while polydispersity of  $> 0.3$  indicates heterogeneity [46]. The particle charge was quantified as zeta potential ( $\zeta$ ). Zeta potential was determined by photon correlation spectroscopy using a Zetaplus (Brookhaven Instruments). The samples were diluted three-fold and assessed in triplicate.

### *2.2.3 HPLC-UV detection of EGCG*

Detection of EGCG was assessed using reverse phase HPLC methodology. A Waters Alliance separation module HPLC with UV detection was utilised at an operating wavelength of 275 nm [47] with a Waters X select column ( $5\text{ }\mu\text{m}$  C18  $4.6 \times 150\text{ mm}$ , 186005290), with a  $10\text{ }\mu\text{L}$  injection volume. The mobile phase comprised of a 70:30 ratio of 0.1% TFA in water to methanol at a flow rate of  $1\text{ mL/min}$ . Stock solutions and standard solutions of EGCG were prepared with both water and ethanol ranging from  $0.5\text{-}500\text{ }\mu\text{g/mL}$ . A final calibration curve with an  $R^2$  of 0.997 and linear equation of  $y = 1 \times 10^7 \cdot x$  was obtained.

### *2.2.4 Entrapment efficiency of EGCG*



The entrapment efficiency of EGCG loaded deformable liposomes was determined by centrifuging samples and quantifying the EGCG in the supernatant. Samples were centrifuged at 18,000 rpm for 30 min at 4°C in an Optima<sup>tm</sup> MAX-XP ultracentrifuge to separate the incorporated drug from the free drug. The supernatant was then analysed using HPLC to determine the encapsulation efficiency of EGCG in liposomal formulations (Equation 1):

$$E = \frac{D_t - D_s}{D_t} \times 100\% \quad (1)$$

where  $E$  is the encapsulation efficiency (%),  $D_t$  is the total drug content (mg) and  $D_s$  is drug content in supernatant (mg).

#### 2.2.5 Assessment of liposomal deformability

To assess the deformability of formulated liposomes, a liposome suspension (6 mL) consisting of a 16:8 micromolar ratio of PC to cholesterol formulated with up to 10% w/w of Tween 20 solution (diluted 3 fold), was passed through a polycarbonate filter of 50 nm pore size using a syringe driver (Cole Parmer, UK) set at 0.6 mL/min for 10 min. The mean particle size and the polydispersity index of liposomes were subsequently quantified by DLS, before and after filtration, to assess the ability of formulated liposomes to regain their size after having been forced through a pore size smaller than their original diameter. The deformability was quantified through the calculation of a deformability index (equation 2) [32]:

$$D = 100 - \frac{L_e}{L} \times 100 \quad (2)$$

where  $D$  is deformability,  $L_e$  is size of extruded liposomes (nm),  $L$  is size of liposomes (nm) prior to extrusion.

#### 2.2.6 Differential scanning calorimetry of EGCG and EGCG lipid blends

To assess thermal characteristics of materials including melting temperatures, phase transitions and heat capacity changes of liposomes, EGCG and ratios of lipid, surfactant and drug mixtures corresponding to that of the liposome formulation, were analysed in the solid state using a TA Instruments Q200 Thermal Analysis Differential scanning calorimetry (DSC). 3 mg of EGCG was weighed into T-Zero aluminium pans and then hermetically sealed. All experimental runs commenced at an initial temperature of 0°C, purged under nitrogen gas, with a scan rate of 10°C/min to 300°C.

#### *2.2.7 In-vitro EGCG release studies*

To assess the impact of inclusion of Tween 20 on EGCG from liposomal formulations, a side—by-side diffusion cell (PermeGear diffusion cell, Hellertown, USA) was maintained at 35 °C. Release was assessed over a 24 hour period from an EGCG aqueous solution (0.1 mg/mL) and EGCG-loaded liposomes (final loading for liposomes formulated with 0, 2, 6 and 10 % w/w Tween 20 was 0.80, 0.55, 0.17 and 0.04 mg/mL respectively). 10 mL of each formulation was placed into the donor side of the diffusion cell and release across a 50 nm membrane (Whatman®) into the receiver side containing 100 mL of dermal dissolution media with a stirrer was measured. The release media was sampled with volume replacement (0.5 mL) over 24 hours and analysed using HPLC-UV quantification.

#### *2.2.8 In-vitro drug release kinetics*

Several kinetic drug release mathematical models were used to assess drug release from the formulations. The best-fit to the mathematical models described below confirmed the appropriate release kinetics:

$$\text{Zero order model: } \frac{M_t}{M_\infty} = k_0 \cdot t \quad (3)$$

where  $M_t/M_\infty$  is the drug fraction released at time  $t$  and  $k_0$  is the zero-order release constant.

210 *First order model:*  $\frac{M_t}{M_\infty} = 1 - e^{-k_1 t}$  (4)

211 where  $M_t/M_\infty$  is the drug fraction released at time  $t$  and  $k_1$  is the first-order release constant.

212 *Higuchi model:*  $\frac{M_t}{M_\infty} = k_H \cdot t^{\frac{1}{2}}$  (5)

213 where  $M_t/M_\infty$  is the drug fraction released at time  $t$  and  $k_H$  is the Higuchi constant.

214 *Korsmeyer-Peppas Model:*  $\frac{C_t}{C} = K t^n$  (6)

215 where  $C_t/C$  is fraction of drug released at time  $t$ ,  $k$  is the release rate constant. The value of  $n$   
 216 is valuable in understanding drug release mechanisms. When  $n \leq 0.45$  drug release is  
 217 diffusion controlled and can be referred to as ‘Fickian’ diffusion and when  $n > 0.89$  the  
 218 diffusion is indicative of erosion controlled drug release or class-II kinetics. For situations  
 219 where  $0.45 < n \leq 0.89$  the diffusion is a complex mixture of both processes and often termed  
 220 anomalous transport. In all cases this is based on the assumption of release from a cylinder  
 221 and applied to cumulative release rates < 60 % [48]

222 Mathematical models to assess release kinetics were fit using Microsoft Excel®. Zero order,  
 223 first order, Higuchi and Korsmeyer-Peppas release profiles were applied to release from drug  
 224 solution and drug loaded liposome solution following which regression analysis techniques  
 225 were employed to determine the probable drug-release. The release kinetic model displaying  
 226 the highest  $r^2$  metric ( $\geq 0.95$ ) was determined to be the mechanism, by which release occurred.

### 227 2.2.9 Liposome stability

228 The stability of liposomes was determined, as prepared in water, through the assessment of  
 229 particle size over a 28-day period, stored in a stability cabinet maintained at  $25 \pm 2$  °C  
 230 (Firlabo, France) at a humidity of  $60 \% \pm 5 \%$ . Mean particle sizes were determined on days

1, 2, 7, 14, 21 and 28 by DLS. Furthermore, the encapsulation efficiency of drug loaded liposomes was assessed over 4 weeks as detailed in section 2.2.4.

#### *2.2.10 Development of an in-vitro cellular dermal model*

To develop an *in-vitro* system to assess cellular toxicity and cellular uptake of deformable liposomes into representative human dermal tissue, two dermal cell line were examined. Human dermal fibroblasts (HDFa) were cultured in Medium 106 supplemented with low serum growth supplement. Human epidermal keratinocytes (HaCaT) cells were revived and sustained in high glucose supplemented DMEM media. Media was replaced every 3 days. At 70-80% confluency, media was discarded and cells detached using Trypsin/EDTA incubated for 5 min, prior to trypsin neutralisation with 3 mL growth media and subsequent centrifugation at 1200 rpm for 10 min and resuspension in fresh media prior to being utilized for subsequent studies

#### *2.2.11 Cellular toxicity of liposomal formulations towards HDFa and HaCat cells*

To determine the cytotoxicity profile of EGCG towards HDFa and HaCat cells, a (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT) assay [49] was performed to measure cell viability after exposure to increasing concentrations of EGCG for 24 hours. Cells were seeded at a density of  $50 \times 10^3$  cells per well into a 96-well plate and grown for 3 days. Thereafter, media was removed and cells were exposed to 100  $\mu$ L of 0.1-100  $\mu$ M EGCG and incubated for 24 hours at 37°C. Subsequently, 25  $\mu$ L of a 12.5:1 (XTT: menadione) was added each well and incubated for 3 hours at 37°C prior to the absorbance being read at 450 nm. Assessment of EGCG toxicity to these cells was conducted through analysis of changes in XTT absorbance with increasing drug concentration.

#### *2.2.12 Intracellular uptake of deformable liposomes into HDFa and HaCat cells*

Liposomes, both deformable and non-deformable, were formulated with the addition of 0.25 mL of a 0.1 mg/mL DilC during the lipid mixing stage. Untrapped DilC was removed by centrifuging liposomes at 18,000 g for 30 min. Coverslips were coated for 30 min with poly-l-lysine (0.01 % w/v) prior to the addition of cells at a density of  $50 \times 10^3$  cells per coverslip. After 24 hours, DilC loaded liposomes were diluted with 1 part of supplemented media (as clarified in materials) and were then added to the coverslips and incubated for 2 hours at 37°C.

Thereafter, coverslips were washed and fixed with 4 % w/v paraformaldehyde for 5 minutes at room temperature. Subsequently, coverslips were mounted onto glass slides with the addition of a DAPI-containing mounting media. Cover slips were subsequently analysed in an upright confocal microscope (Leica SP5 TCS II MP) and visualised with a 40× oil immersion objective. Images were acquired using a helium-neon laser at 633 nm to visualise DilC and a helium–neon laser to visualise DAPI at 461 nm.

#### *2.2.13 Statistical analysis*

Unless otherwise stated, all results are presented as mean  $\pm$  standard deviation (SD). Replicates of at least 3 were used for all studies. For multiwell plate assays replicates of 6 were used for each experimental condition with the study replicated 3 times. A one-way ANOVA was used to determine any statistically significant difference between means tested ( $p \leq 0.05$ ). A post-hoc Tukey's multiple comparisons test was then applied to assess differences between groups. All the calculations were carried out using Graphpad 6 (GraphPad Inc., La Jolla, CA).

### **3. Results and discussion**

276 Emerging treatments for cancer management involve chemoprevention and chemoprotection.  
277 Current anticancer agents tend to demonstrate a poor safety profile in addition to possess a  
278 wide range of unpleasant side effects [10-12]. However, phytochemical flavonoids, such as  
279 EGCG, are increasingly being investigated for their chemoprevention properties [13-15].

280 EGCG is a flavonoid found in green tea that possesses cytotoxic effects in cancerous skin  
281 cells and thus may be a potentially viable candidate as a pharmacological anti-cancer agent  
282 [16], given that it has been observed to induce apoptosis in cancer cells without affecting  
283 normal cells [50, 17], in addition to the modulating expression of a number of genes involved  
284 in cell proliferation, cell-cell contact and cell-matrix interactions [51].

285 However, the penetration of drugs across the skin is significantly hindered by the skin's  
286 inherent barrier properties [25]. The use of deformable liposomes to aid dermal cellular  
287 penetrability and uptake may be advantageous in the targeting of neoplastic agents to deeper  
288 skin cellular layers when compared to conventional liposomes which may not be able to  
289 penetrate through the narrow pore of the stratum corneum [39].

290 This focus of this study was to develop EGCG loaded slow release deformable liposomes.  
291 EGCG liposomes were formulated with PC and cholesterol with the inclusion of Tween 20 as  
292 a edge-activator with incorporation of up to 10 % w/w. Liposomal characteristics including  
293 liposome size, charge, encapsulation efficiency, DI, release profile, stability, ceulluar toxicity  
294 and uptake were was assessed.

### 295 ***3.1 Liposome characterisation***

296 The impact of the inclusion of Tween 20 within the liposomal formulation on  
297 liposome characteristics were observed. As the surfactant loading in the bilayer of 'empty'  
298 liposomes increased, liposome diameter decreased from  $206 \pm 24$  nm for liposomes

formulated with no surfactant to  $102 \pm 11$  nm for liposomes formulated with 10 % w/w Tween 20 (Figure 1A). As the surfactant loading in the bilayer of EGCG loaded liposomes increased, liposome diameter decreased, from  $258 \pm 17$  nm for liposomes formulated with no surfactant compared with  $105 \pm 13$  nm for liposomes formulated with 10 % w/w Tween 20 (Figure 1B). The decrease in size was statistically significant ( $p \leq 0.05$ ) for ‘empty’ and EGCG loaded liposomes formulated with no surfactant compared with liposomes loaded with 2, 6 and 10 % w/w Tween 20.

[Figure 1 near here]

The inclusion of surfactants into liposome formulations have previously been demonstrated to decrease liposome size when compared to liposomes formulated in the absence of surfactant [26, 32]. This may be as a result of a destabilising effect imparted by the surfactant on the bilayer [52], which results in a greater interaction of the phospholipid bilayer with the aqueous phase. A consequence of this would then be the overall formation of liposomes with a smaller diameter giving a greater surface area in contact with the aqueous phase. The inclusion of surfactant has been previously reported to decrease liposome size in comparison to conventional liposomes. A study formulating liposomes with Phospholipon® 90 G and both Tween 80 and Span 80 reported a size reduction from 207 nm to 139 nm following inclusion of the surfactants [32].

A liposome preparation which is homogenous in size is important as final liposome size will partly determine the level of tissue distribution *in-vivo* in addition to influencing drug release kinetics. A polydispersity of up to 0.3 is considered homogenous [53, 32, 54]. As the loading of surfactant increased, the polydispersity of the liposomal formulation did not significantly change for ‘empty’ liposomes (Figure 1a) whilst a significant decrease was observed for those loaded with EGCG ( $P \leq 0.0001$ ) from 0.3 to 0.2 (Figure 1b). Therefore,

the inclusion of Tween 20 within the liposome formulation appeared to improve homogeneity (although liposome production method may also impact this quality).

The magnitude of the zeta potential ( $\zeta$ ) indicates the degree of electrostatic repulsion between adjacent, similarly charged particles in a dispersion. Thus, it is a fundamental parameter thought to affect stability of liposomal formulations. All formulated liposomes demonstrated a near neutral charge (Table 1). A neutral liposomal surface charge is important to avoid skin irritation [55] however, this may subsequently lead to particle flocculation due to a lack of electrostatic repulsion between liposomes causing them to cluster [56].

[Table 1 near here]

In liposomes formulated with EGCG, as Tween 20 loading increased, a statistical significant decrease in EGCG entrapment was observed, ( $P \leq 0.0001$ ), from  $80.0 \pm 3.0 \%$  when no surfactant is present to  $4.3 \pm 3.0 \%$  with a 10 % w/w loading of surfactant (Figure 2). This decrease in EGCG loading may be related to the difference in the molecular weight of EGCG and Tween 20. Tween 20, with larger molecular weight of 1227.54 g/mol compared to that of EGCG (386.65 g/mol), may be assumed to be better poised to displace EGCG from the bilayer. Further, the hydrophobic tail of Tween 20 would have a high affinity to the chains in PC disrupting the liposome structure as well as displacing EGCG from the bilayer [57-59]. Furthermore, Tween 20 is known to enhance the solubility of drugs and therefore, as not all would be entrapped within the bilayer, this may allow increased EGCG solubilisation within the liposomal rehydration media [60]. It is also possible that the coexistence of vesicles and mixed micelles at high surfactant concentrations [61] may have reduced the compound entrapment in mixed micelles.

[Figure 2 near here]



The degree of deformability of each formulation was determined by extrusion through a polycarbonate filter with a pore size of 50 nm. The mean particle size and the polydispersity index of liposomes was quantified before and after filtration to assess liposome ability to regain size after having being forced through a pore size smaller than their original diameter. The DI is defined as the degree the liposomes deformed; the greater the degree of deformation the less elastic the liposomes are as they were unable to regain their previous larger size. The DI following extrusion decreased with statistical significance ( $P \leq 0.0001$ ) as surfactant loading increased in ‘empty’ liposomes, from  $71 \pm 6$  for liposomes formulated with no surfactant compared with  $26 \pm 3$  % for liposomes formulated with 10 % w/w Tween 20 respectively. EGCG liposomes formulated with Tween 20 demonstrated a statistically significant decrease ( $P \leq 0.0001$ ) in DI from  $74 \pm 8$  for liposomes formulated with no surfactant compared with  $37 \pm 7$  for liposomes formulated with 10 % w/w Tween 20 (Figure 3). These observations imply the liposomes were displaying elastic properties as they could deform in order to pass through an opening smaller than its own diameter whilst, to a certain degree regaining its size. It is important to address the initial size of liposome. Those formulated with Tween 20 were of a smaller diameter thus would require less deformation to fit through the pores. However, the pore size was smaller than liposome diameter in all cases and the DI equation requires the ratio of pre to post extrusion size. Additionally, the presence of EGCG in the liposome formulation did not appear to affect the DI compared with liposomes formulated without. A study formulating liposomes with Phospholipon® 90 G and both Tween 80 and Span 80 saw a size reduction observed surfactant to decrease the DI from  $51 \pm 4$  to  $17 \pm 5$  [32].

[Figure 3 near here]

Liposomes formulated with surfactant can deform as the surfactant has a propensity for highly curved structures (e.g. micelles and liposomes), thus diminishing the energy required for particle deformation. The surfactant is able to diminish the energy required for particle deformation and accommodate particle shape changes of the liposomes under stress [62]. These surfactants may have interacted with the PC with strong affinity but in reversible mode. The reversible binding mode might have provided the deformability upon physical stress [38].

For liposomes to deform, a source of energy is required [63-65]. In our systems, 'energy' was supplied to this system in the form of pressure as a result of the action of the syringe driver. The larger the concentration of surfactant included within the formulation, the greater the energy the liposome as a whole is able to retain [65]. It is postulated that this energy is used to reorientate the lipid bilayer structure, and since all systems tend toward the lowest state of free energy, the energy stored in this structure will be expelled once the liposome has passed through the pore and there is no longer any pressure forcing the bilayer to remain in an 'unnatural state' [35, 36, 66]. This energy can then be expended into reforming the liposome. Some energy will be lost during passage as heat or non-plastic deformation, therefore it was not possible to attain a DI of 0 %.

The energy used to alter the bilayer of a liposome containing no surfactant does not benefit from the extra 'storage space' of a surfactant, thus energy may be expended to rupture the membrane causing liposome size to decrease [65]. Despite the potential for excess energy in liposomes formulated with Tween 20, liposomes were not able to fully regain their pre-extrusion size. Some energy will always be lost in the friction of the particles moving through the pores as heat [67]. An increase in surfactant loading may bring the liposomes closer to 100% reformation [65]. Further, liposomes unable to fit through the pores or lipid aggregates

from ruptured liposomes may cause blockages. This may lead to an increase in pressure in the vessel causing more turbulence leading to the rupture and non-uniform reformation of liposomes. Additionally, *in-vivo*, liposomes would be expected to move across the skin following an osmotic transepidermal gradient as has been reported in many similar studies concerning the dermal and transdermal delivery of drug [64, 39, 65, 32]. Such lipid carriers are miscible with the epidermal lipids present within the barrier of the stratum corneum thus would be able to penetrate into deeper layers of the skin [68-70]. Furthermore, the skin is warmer than room temp (35 °C compared to 20 °C). Temperature governs the energy term of enthalpy therefore the liposomes would have more energy to be even more flexible and cross the stratum corneum.

### **3.2 Differential scanning calorimetry investigations of EGCG and EGCG lipid blends**

Differential scanning calorimetry (DSC) has been widely used in understanding the thermal characteristics of materials where an insight into a range of thermal properties including melting temperatures, phase transitions and heat capacity changes can be obtained. It has been observed that drugs with melting point of < 200 °C are better poised to cross the SC [71, 24], therefore observing the effect of formulation parameters on the melting point would aid formulation development. The glass transition temperature ( $T_g$ ) of EGCG was identified at 220 °C (peak c) and the melting point ( $T_m$ ) of EGCG was at 245 °C (peak d) (Figure 4) and concurred with those reported by Cho et al (2008) where the  $T_m$  of GCG (an epimer of EGCG) was at 223 °C, the  $T_g$  of EGCG was at 235 °C and the  $T_m$  of EGCG was at 246 °C. Cho et al also observed a peak at 97 °C and determined it to be the conversion temperature of EGCG into GCG. Therefore, the first two troughs (peak a and b) observed in the scan may be representative of the epimer GCG [72]. Differences between reported values and those observed here may be due to differences in EGCG sample purity.

[Figure 4 near here]

The DSC of the lipid (PC and cholesterol) and Tween 20 blend observed the  $T_m$  of this mixture to be 172 °C (Figure 5A). There is also a smaller absorption peak at 208°C which would be attributed to one of the liposomal components. Upon addition of EGCG to this mixture, comparison to the aforementioned DSC data observed the melting point shifted to 191 °C (Figure 5B). Furthermore the  $T_m$  of EGCG decreased from 242°C to 191°C illustrating that the surfactant loaded liposomes could decrease the  $T_m$  of EGCG thus potentially improving partitioning across a membrane [73-75]. Furthermore, the addition of EGCG caused the peak at 208°C to either disappear or shift into the main peak observed at 191°C.

[Figure 5 near here]

### ***3.3 EGCG release studies from liposomal formulations***

The release of EGCG from solution and liposomal formulations was studied over a 24-hour period (Figure 6). Liposomes appeared to retard the release of EGCG in comparison to release across the membrane from EGCG in solution. Furthermore, with increasing the loading of Tween 20 within liposomal formulations (0 to 10 % w/w), EGCG release increased from  $14 \pm 1$  % at 24 hours for 0 % w/w Tween 20, to  $94 \pm 5$  % at 24 hours for 10 % w/w Tween 20. The cumulative percentage released after 24 hours was significant between the solution and liposomes loaded with 0%, 2%, and 6% w/w of Tween 20 ( $P \leq 0.0001$ ). The inclusion of surfactant enables an increase in drug solubility of poorly soluble compounds thus explaining the increase in drug release at higher loadings of surfactant. Such properties are already exploited to improve the oral delivery release profiles of poorly soluble compounds in self-emulsifying drug delivery systems with four drug products [76, 77], Sandimmune® and Sandimmun Neoral® (cyclosporin A), Norvir® (ritonavir), and

Fortovase® (saquinavir) on the pharmaceutical market [76]. It is worth noting that as surfactant loading increased, EGCG entrapment decreased thus a lower concentration gradient would be observed. This did not appear to retard EGCG release.

[Figure 6 near here]

An increased rate of release was observed from the EGCG solution compared with liposome formulations over the 24 hours observed (Table 2). Further, as the loading of surfactant increased, the rate of EGCG release increased (from  $0.034 \pm 0.013$  to  $0.993 \pm 1.013$  for liposomes loaded with 0 % and 10 % of Tween 20 respectively based on the Korsmeyer-Peppas model). Thus, surfactant appears to increase drug release/encourage liposome breakdown, particularly at 10 % w/w where the rate was 10 fold greater than that at 6% w/w. The surfactant would increase drug solubility thus explaining why an increase in drug release is observed at higher loadings of surfactant. The inclusion of surfactant destabilizes the vesicle bilayers by reducing the amount of work required to expand the interface allowing the liposome to become more flexible [40, 38, 39] and move through the membrane. Additionally, it has been suggested that the mechanism of the *in-vitro* release seems to be the formation of transient pores in the lipid bilayer, through which drugs are released to the extra-liposomal medium (Wang, Wang et al. 2016).

[Table 2 near here]

Based on the values of the determination coefficient ( $R^2$ ), as well as AIC values (Akaike Information Criterion), the model that best describes EGCG release from all liposomal formulations is Korsmeyer-Peppas model (highest  $R^2$  and lowest AIC). The diffusion release exponent value revealed a range of release mechanisms for each formulation. Liposomes formulated with 0%, 6 % and 10% w/w Tween 20 had an exponent value of  $0.839 \pm 0.072$ ,  $0.836 \pm 0.116$  and  $0.722 \pm 0.247$  respectively indicating the release is a complex

mixture of the diffusion (flux due to molecular diffusion and the concentration gradient) and erosion controlled drug release or class-II kinetics (diffusion not based on concentration gradient) processes and often termed anomalous transport [78]. Liposomes formulated with 2 % w/w Tween 20 observed an exponent value of  $0.913 \pm 0.186$  indicative of erosion controlled drug release or class-II kinetics [48].

### ***3.4 Stability of EGCG loaded deformable liposomes***

The impact of long-term storage of EGCG-loaded liposomes formulated with 2 % w/w Tween 20 was assessed during storage in stability cabinets maintained at  $25 \pm 2$  °C (Firlabo, France) at a humidity of  $60 \% \pm 5 \%$ . Liposomes formulated with 2% w/w Tween were selected in this study as it had the highest EGCG entrapment compared with the higher loadings of surfactant thus will be taken forward for cell uptake studies. The impact of this storage on size (Figure 7) and encapsulation efficiency (Figure 8) was assessed. EGCG loaded liposomes formulated with and without surfactant maintained a consistent size over time (Figure 7) with no statistically significant difference in size during the storage period. Previous reports have highlighted that aggregation is common upon liposomal formulation storage, and results in vesicle size growth [79] particularly, though not always, with neutral liposomes [56]. However, the inclusion of Tween 20 into the deformable liposomes may have prevented this phenomenon and may be a result of surfactant destabilising the lipid bilayer and reducing the energy required to expand the interface, thus allowing maintenance of smaller structures. It appears the inclusion of surfactant prevents this phenomenon which correlates with similar studies [80].

[Figure 7 and 8 near here]

Furthermore, encapsulation efficiency showed a trend of decreasing loading from  $43 \pm 7 \%$  to  $42 \pm 12 \%$ ,  $39 \pm 9 \%$ ,  $30 \pm 11 \%$  to  $30 \pm 6 \%$ , for liposomes formulated with 2 % w/w Tween

20 (Figure 8). Tween 20 is able to increase compound solubility, therefore, this may allow EGCG to solubilise within the liposomal media [60] and leach out over time.

### ***3.5 Cellular toxicity of liposomal formulation towards HDFa and HaCat cells***

Whilst topical formulations are applied directly into the skin, various connective layers making up the skin are important for drug delivery. The skin primarily consists of the epidermis, dermis and subcutaneous layers and each layer has a unique combination of cells, connective tissue, components and functions. Skin cancers develop in the upper layers of the skin spanning the dermal and epidermal layer, and any formulation system should consider the impact of formulation systems on these tissue layers for the delivery of drugs.

In order to assess cellular toxicity of EGCG to these cells, we adopted two *in-vitro* cell culture systems, namely human keratinocyte and human fibroblast cells. To determine the cellular viability cytotoxicity of EGCG towards HDFa and HaCat cells, an XTT assay was performed to measure cell death after exposure of cells to different concentrations of drug for 24 hours (Figure 9).

[Figure 9 near here]

As the concentration of EGCG was increased from 0.1 to 100  $\mu$ M, HDFa cell viability decreased (Figure 9A) with statistical significance ( $P \leq 0.0001$ ). This may be due to toxicity or death of damaged cells in which EGCG induced apoptosis [81, 82]. Whilst limited data exists on the cytotoxicity of EGCG towards dermal tissues, a study observing growth inhibition in multiple cell lines, observed that EGCG at 40  $\mu$ M had little or no inhibitory effect on the growth of WI38 cells, normal human fibroblast cells [83]. Cell viability was maintained across the concentration range of 0.1–100  $\mu$ M on HaCat cells (Figure 9B). No statistically significant difference was observed in cell viability. Furthermore, EGCG has

been reported to impart protective effects in HaCat cells exposed to external stressors including UVA and UVB radiation [84, 85]. Whilst some of our formulations exceeded this concentration of EGCG as a whole, the retarded release profile of the liposomes would be expected to result in an overall lower temporal concentration profile exposure to these cells, significantly below 100  $\mu$ M.

### ***3.6 Cellular liposomal uptake assay into HDFa and HaCat cells***

A primary goal for our studies was to demonstrate uptake of deformable liposomes loaded with EGCG into a cell culture skin model. EGCG loaded liposomes were incubated with both HaCat (Figure 10) and HDFa (Figure 11) cells to assess the cellular uptake of these formulations. Liposomes formulated with 2% w/w Tween 20 were selected, a result of the highest EGCG entrapment compared with the other surfactant loadings. Fluorescently labelled liposomes loaded with EGCG incubated for 2-hours with both HaCat and HDFa cells seeded onto collagen-coated coverslips and the cellular localisation of these liposomes was determined using confocal microscopy. Following a 2-hour incubation with the cells, intracellular localisation of labelled liposomes were clearly evident, confirming the successful uptake into both HaCat and HDFa cells.

[Figure 10 and 11 near here]

Extraneous particle cell uptake is dependent upon influences such as particle size, charge, affinity etc. [86-88]. There are four proposed methods of liposome uptake into cells: stable adsorption, endocytosis, fusion of the lipid bilayer with the cell plasma membrane and lipid transfer [43, 89]. It is unclear which of these, or another process, occurred in this study, however, these methods of uptake are not mutually exclusive and any combination may occur in a given experimental circumstance [43]. The interaction of nanoparticles with cell membrane seems to be most affected by particle surface charge. The cell membrane surface



is dominated by negatively charged sulphated proteoglycans molecules (vital in cellular proliferation and migration) [90, 91]. These molecules are associated with glycosaminoglycan side chains (heparan, dermatan, keratan or chondrotine sulfates) which are anionic, and interaction between proteoglycans and liposomes, if positively charged, tend to be largely ionic [92]. The liposomes applied to the cells in this study had a  $\zeta$  of  $4 \pm 1$  suggesting an ionic interaction may have occurred. A study applying cationic liposomes formulated with the cationic lipids Lipofectin, Tfx-50, and Lipofectamine in oligonucleotide delivery to HaCat cells observed liposome uptake within 24 hours [93]. Furthermore, research developing chemotherapy against malignant melanoma using mouse B16 melanoma cells as well as Normal Human Dermal Fibroblasts observed a greater uptake of cationic liposomes by cells in the injection site compared with neutral liposomes due to the electrostatic interaction with the negative-charged phospholipid membrane of cells [94].

It should be noted that the confocal microscopy studies demonstrated the possibility of the delivery of deformable liposomes to relevant dermal tissues using *in-vitro* cell culture techniques. However, the application of such formulations could also be assessed using *ex-vivo* human or animal dermal tissues. The ultimate aim of this delivery system was to improve dermal cell uptake and delivery a controlled release of active agent, thus from a regulatory perspective, pharmacokinetic data is not required as drug is not intended to reach the blood stream [95].

This study did not address skin penetration. The liposomes formulation recommended to take into further studies would be that loaded with 2% w/w Tween 20. In order to ascertain the extent of carrier and drug permeation a skin strip test may be appropriate [70]. This involves the use of an adhesive tape to strip the skin layer by layer and quantifying lipid and drug on each layer[96]. Further, whilst the most appropriate animal model for human skin is the

porcine skin tissue, sample-to-sample variability in addition to differences in the lipid dermal matrices often results in an altered permeability profile limiting the wider human translational goals [97-99]. The use of skin tissue samples would also help determine whether intact liposomes deliver EGCG to dermal cells or if EGCG delivery is as a result of liposomes being mixed with lipids present in the stratum corneum.

#### 4. Conclusion

Skin cancer is emerging as an increasing public health problem particularly in developed countries. Current treatments include surgery to remove the tumour as well as topical formulations. Such treatments may not be suitable for all patients as they are associated with an unpleasant aesthetic profile as well as side effects. A nanoparticle delivery system such as deformable liposomes applied topically for the direct dermal delivery of compounds would be valuable in carrying compounds across the stratum corneum at a controlled rate whilst limiting side effects. The use of naturally occurring compounds such as EGCG have been found to be successful as chemopreventative and chemoprotective agents. However, formulation of such compounds has been limited in success due to a limited bioavailability of promising agents and inefficient delivery systems. We developed a novel deformable liposome formulation loaded with EGCG and systemically investigated the loading, uptake and *in-vitro* release of EGCG from these nanoparticles. This study has found deformable liposomes could be valuable in targeted delivery whilst offering controlled release of the compound [13, 100]. We have demonstrated that as the amount of Tween 20 in the liposomal bilayer is increased, liposome size decreased and elasticity increased. As the loading of Tween 20 in the liposome was increased the EGCG encapsulation decreased. This may have been due to Tween 20 competing for space within the bilayer or due to Tween 20 increasing the solubilisation capacity of EGCG. Additionally EGCG release from liposomes

found that the liposomes were able to modify the release of drug with complete release observed within 24 hours. Further, our studies demonstrated these liposomes were capable of uptake into epidermal keratinocytes and dermal fibroblasts within 2 hours. This present study demonstrates liposomes formulated with Tween 20 are useful in the development of a controlled release topical formulation for dermal delivery crucial in improving patient compliance thus skin cancer treatment outcomes.

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848 **Table 1:** Zeta potential of liposomal formulations formulated in the absence and presence of  
 849 up to 10% w/w of Tween 20

Surfactant loading (% w/w)	Zeta potential (mV)	
	'empty'	EGCG loaded
	liposomes	liposomes
<b>0</b>	5.03 ± 1.03	2.41 ± 1.08
<b>2</b>	4.67 ± 1.08	3. 67 ± 0.91
<b>6</b>	3.71 ± 0.90	-0.99 ± 1.01
<b>10</b>	-2.79 ± 0.20	-1.90 ± 0.88

850 Results are presented as the mean ± standard deviation (n=3)

851



852 **Table 2:** *In-vitro* SA release kinetics models

Kinetic model	Parameter	Tween 20 Loading (% w/w)			
		0	2	6	10
<b>0</b>	$(k_0) \times 10^{-2}$ $\text{mg} \cdot \text{min}^{-1}$	$1.01 \pm 0.05$	$1.18 \pm 0.01$	$2.75 \pm 0.05$	$7.77 \pm 0.02$
	$R^2$	$0.95 \pm 0.02$	$0.99 \pm 0.01$	$0.93 \pm 0.07$	$0.72 \pm 0.30$
	AIC	$28.59 \pm 4.44$	$18.82 \pm 2.96$	$57.24 \pm 14.21$	$98.21 \pm 25.32$
<b>1<sup>st</sup></b>	$(k_1) \times 10^{-4}$ $\text{min}^{-1}$	$1.07 \pm 0.05$	$1.27 \pm 0.13$	$3.32 \pm 0.58$	$15.04 \pm 3.11$
	$R^2$	$0.96 \pm 0.02$	$0.986 \pm 0.01$	$0.96 \pm 0.04$	$0.94 \pm 0.06$
	AIC	$25.68 \pm 4.40$	$18.132 \pm 6.83$	$54.03 \pm 6.46$	$4.56 \pm 17.86$
<b>Higuchi</b>	$k_H$	$0.26 \pm 0.01$	$0.294 \pm 0.03$	$0.71 \pm 0.09$	$2.12 \pm 0.14$
	$R^2$	$0.83 \pm 0.04$	$0.7513 \pm 0.03$	$0.81 \pm 0.05$	$0.86 \pm 0.10$
	AIC	$47.40 \pm 5.92$	$58.783 \pm 4.90$	$77.32 \pm 9.63$	$99.05 \pm 13.13$
<b>Korsmeyer-Peppas</b>	$k_{KP}$	$0.03 \pm 0.01$	$0.059 \pm 0.08$	$0.09 \pm 0.06$	$0.99 \pm 1.01$
	N	$0.84 \pm 0.07$	$0.913 \pm 0.19$	$0.84 \pm 0.12$	$0.72 \pm 0.25$
	$R^2$	<b><math>0.99 \pm 0.01</math></b>	<b><math>0.991 \pm 0.01</math></b>	<b><math>0.99 \pm 0.01</math></b>	<b><math>0.96 \pm 0.03</math></b>
	AIC	$11.26 \pm 3.00$	$27.163 \pm 22.24$	$49.82 \pm 8.49$	$82.76 \pm 9.81$

853  $R^2$ , coefficient of determination; AIC, Akaike Information Criterion; F is the fraction of drug released at time t;  $k_0$  is the zero-order release  
854 constant;  $k_1$  is the first-order release constant;  $k_H$  is the Higuchi release constant;  $k_{KP}$  is the release constant incorporating structural and  
855 geometric characteristics of the drug- dosage form; n is diffusion release exponent.

## 856 List of figures

### 857 **Fig. 1** Liposome size distribution and polydispersity of ‘empty’ and EGCG loaded liposomes

858 Liposome size distribution and polydispersity, determined by DLS, comparing (A) ‘empty’  
859 and (B) EGCG loaded formulations with Tween 20 (0-10 % w/w). Liposomes were prepared  
860 by the dry film hydration method and EGCG added during the lipid mixing stage. Data  
861 represents mean  $\pm$  SD. n=3 independent batches. \*\*\*\* indicates statistical comparison  
862 between the size of liposome formulations with a  $P \leq 0.0001$ . # # indicates statistical  
863 comparison between the polydispersity of liposome formulations with a  $P \leq 0.01$ .

### 864 **Fig. 2** Entrapment efficiency of EGCG in liposomes formulated with 0-10% w/w Tween 20

865 Entrapment efficiency (%) of EGCG in liposomes formulated with varying amounts of  
866 Tween 20 (0-10% w/w) Data represents mean  $\pm$  SD. n=3 independent batches. \*\*\*\* indicates  
867 statistical comparison between the entrapment efficiency of liposome formulations with a  $P \leq$   
868 0.0001.

### 869 **Fig. 3** Deformability index for ‘empty’ and EGCG loaded liposomes

870 Deformability index following extrusion through 50 nm membranes for ‘empty’ and EGCG  
871 loaded liposomes with increasing surfactant loading up to a maximum of 10% w/w.  
872 Liposomes were prepared adapting the dry film method adding the surfactant and adding  
873 EGCG during the lipid mixing stage. The preparation was vortexed and then extruded through  
874 the membranes. Data represents mean  $\pm$  SD. n=3 independent batches. \*\*\*\* indicates  
875 statistical comparison between the DI of liposome formulations with a  $P \leq 0.0001$ .

### 876 **Fig. 4** Differential scanning calorimetry scan of EGCG

877 All experimental runs commenced at an initial temperature of 0 °C with a scan rate of 10  
878 °C/min to 300 °C. Peak a and b are related to the epimer of EGCG, GCG. Peak c represents  
879 the glass transition temperature ( $T_g$ ) of EGCG was at 220 °C and the melting point ( $T_m$ ) of  
880 EGCG was at 245 °C.

### 881 **Fig. 5** Differential scanning calorimetry analysis scans of PC, cholesterol and Tween 20 and 882 EGCG blends

883 DSC analysis scans of (A) PC, cholesterol and Tween 20 blend and (B) PC, cholesterol,  
884 Tween 20 and EGCG blend. The  $T_m$  of the lipid mixture is 172 °C, and upon addition of  
885 EGCG, the  $T_m$  was 191 °C. All experimental runs started at an initial temperature of 0 °C,  
886 purged under nitrogen gas, with a scan rate of 10 °C/min to 300 °C.

### 887 **Fig. 6** *In-vitro* percentage EGCG cumulative release profiles from solution and liposomal 888 formulations

889 EGCG release profiles from solution and liposomes formulated with 0, 2, 6 or 10 % w/w  
890 Tween 20 over 24 hours. Liposomes were prepared adapting the dry film method adding the  
891 surfactant and EGCG during the lipid mixing stage. A diffusion cell dialysis system was used  
892 to evaluate *in-vitro* drug release. Data represents mean  $\pm$  SD. n=3 independent batches. \*\*\*\*

indicates statistical comparison between the EGCG release of liposome formulations with a  $P \leq 0.0001$ .

**Fig. 7** Stability of EGCG loaded liposomes as determined by size

Size of EGCG loaded liposomes formulated with 0-10% w/w Tween 20, using DLS, formulated with up to 10% w/w Tween 20 measured on various days (1, 7, 14, 21 and 28). Data represents mean  $\pm$  SD. n=6 independent batches.

**Fig. 8** Liposome encapsulation efficiency for EGCG

Liposome encapsulation efficiency for EGCG in liposomes formulated with 2 % w/w Tween 20 liposomes over 28 days. Liposomes were prepared adapting the dry film method adding the surfactant and drug during the lipid mixing stage. The preparation was then washed via centrifugation. The quantity of EGCG in supernatant over 28 days was then analysed by HPLC coupled with UV detection to assess liposome stability. Data represents mean  $\pm$  SD. n=6 independent batches.

**Fig. 9** Cellular toxicity of EGCG

HDFa (A) and HaCat (B) cells were grown on a 96-well plate at a density of  $50 \times 10^3$  cells per well and exposed to various concentrations of EGCG (0.01-100  $\mu$ M) for 24 hours. Thereafter 25  $\mu$ L of a 12.5:1 parts mixture of XTT to menadione was added each well. Plates were incubated for 3 hours at 37°C and the absorbance read at 450 nm. Data is reported as mean  $\pm$ SD with 6 replicates per compound in at 3 independent experiments. \*\*\*\*, \*\*\*, \*\*, \* indicates statistical comparison between the entrapment efficiency of liposome formulations with a  $P \leq 0.0001$ , 0.001, 0.01 and 0.05 respectively.

**Fig. 10** Localisation of DilC labelled liposomes loaded with EGCG and 2% w/w Tween 20 in HaCat cells

Cells were grown on the coverslips for 2 days. Cell nuclei were visualised using (A) DAPI (Blue). Liposomes were formulated with DilC for visualisation (B) (yellow). Liposome localisation within the cell is shown in the merged image (C).

**Fig. 11** Localisation of DilC labelled liposomes loaded with EGCG and 2% w/w Tween 20 in HDFa cells

Cells were grown on the coverslips for 2 days. Cell nuclei were visualised using (A) DAPI (Blue). Liposomes were formulated with DilC for visualisation (B) (yellow). Liposome localisation within the cell is shown in the merged image (C).

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